ROLE OF MATRIX STIFFNESS AND STRESS RELAXATION ON MECHANOTRANSDUCTION OF HaCaT CELLS

A PROJECT SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

Master of Technology

In

Biotechnology

By

SHRUTIJA PANDEY

212BM2014

Under the Supervision of

Dr. INDRANIL BANERJEE

Department of Biotechnology & Medical Engineering

National Institute of Technology

Rourkela-769008, Orissa, India

May, 2014

CERTIFICATE

This is to certify that research project report entitled "**Role of matrix stiffness and stress relaxation on mechanotransduction of HaCaT cells**" submitted by **Shrutija Pandey**, in partial fulfillment of the requirements for the award of the Degree of Master of Technology Biotechnology and Medical Engineering with specialization in Biotechnology at National Institute of Technology Rourkela is an authentic work carried out by him under my supervision and guidance.

To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University/ Institute for the award of any Degree or Diploma.

Dr. Indranil Banerjee

Assistant Professor

Department of Biotechnology and Medical Engineering

National Institute of Technology, Rourkela

ACKNOWLEDGEMENT

If words are reflected as symbols of appreciation and token of acknowledgement, then words play the role of thanks to exhibit the deeply embedded feeling of gratitude. I am greatly indebted to, who either through guidance, discussion or providing facilities for the thesis work, have served as a beacon light or crowned my efforts with success. With an overwhelming sense of pride and genuine obligation I express my deep sense of heartfelt gratitude and regards to my guide **Dr. Indranil Banerjee**, Department of Biotechnology and Medical Engineering, National Institute of Technology Rourkela for giving me an opportunity to do the project work in an independent arena.

I consider it a privilege to express my gratefulness to **Dr. T. K. Maiti** and **Mr. Bibhas Roy**, Department of Biotechnology, Indian Institute of Technology Kharagpur for the valuable guidance and suggestions during the project work.

I sincerely thank the Head of the department, **Prof. K. Pramanik** and all my faculty members for their blessings and guidance in my work.

Further, I would like to express my thankfulness to **Prof. K. Pal** and **Prof. S. K. Bhutia** for their help and providing access to their lab.

I am also thankful to **Mr. A. K. Senthilguru** and **Mr. Sai S. Sagiri** for their constant support and advice in my project work.

Finally, I am grateful to my parents **Mr. Som Prakash Pandey**, **Mrs. Abha Pandey** and friends for their endurance, love, wishes and support, which helped me in completion of my work.

(**Shrutija Pandey**)

TABLE OF CONTENTS

Chapters	Description	Page
Abstract		i
List of Tables & Figures		ii-iii
Chapter 1.	INTRODUCTION	1-3
Chapter 2.	REVIEW OF LITERATURE	4
	2.1 Role of matrix stiffness in cell proliferation and cytoskeletal reorganization	5-6
	2.2 Biophysical hallmarks of cell cycle progression	7-8
	2.3 Nuclear Mechanotransduction	8-10
Chapter 3.	OBJECTIVES AND WORK PLAN	11
	3.1 Objective of the work	12
	3.2 Plan of work	12
	3.3 Rationale of work	13
Chapter 4	MATERIALS AND METHODS	14
	4.1 Materials	14
	4.2 Preparation of PDMS substrates of varying elasticity	14
	4.3 Mechanical characterization of PDMS substrates	15-16
	4.4 Cell culture	16
	4.5 Synchronization procedure	16-17
	4.6 Cellular reorganization	17
	4.7 Immunocytochemistry protocol	17-18
	4.8 Cell cycle analysis through Flow cytometry	18
Chapter 5.	RESULTS AND DISCUSSION	19
	5.1 Mechanical characterization of PDMS substrates	20-21
	5.2 Proliferation study of HaCaT cells cultured over PDMS substrates of varying stiffness	21-22
	5.3 Cell cycle analysis of synchronized HaCaT cells cultured over PDMS substrates of varying stiffness	22-23

	5.4 Expression of pERK1/2 in HaCaT cell cultured over PDMS substrates of varying stiffness	23-24
	5.5 Cytoskeletal reorganization of HaCaT cells on PDMS matrices of varying stiffness	24-25
	5.6 Cell adhesion mediated signalling : expression of $pFAK^{397}$	25-26
	5.7 Nuclear mechanotransduction	26-27
Chapter 6.	CONCLUSION	28
	4.1 Conclusion	29
	4.2 Future perspectives	29
Chapter 7.	REFERENCES	30-33

ABSTRACT

Understanding mechanotransduction appears as an important step in numerous fields of biology, such as cancer, regenerative medicine and tissue engineering. Among the various mechanotransducers, matrix elasticity has been considered as universal regulator of cell behavior. Though, the number of materials that have been investigated is quite small. However, this concept has been recently re-evaluated in the context of other matrix properties such as matrix poroelasticity and stress relaxation along with matrix elasticity. Keeping this in mind here we have investigated the effect of stress relaxation along with matrix elasticity on cell behavior using Polydimethyl siloxane (PDMS) material. For this purpose PDMS matrix of different elasticity and stress relaxation properties (within physiological range) were prepared by varying the base: crosslinker ratio and the effect of the aforesaid physical properties on HaCaT cell physiology were investigated. The study includes cytoskeletal reorganization, proliferation, cell cycle regulation and nuclear mechanotransduction. PDMS matrices were found to have no effect on cell spreading, proliferation and cell cycle regulation of HaCaT cells. The results were further confirmed by checking the expression of FAK-ERK using immunocytochemistry and flow cytometry. However, the variation in matrix stiffness and stress relaxation in case of PDMS matrices were found to be capable of inducing nuclear mechanotransduction as evident from Lamin A/C expression profile. The study showed expression of Lamin A/C varied inversely with PDMS matrix stiffness but maintains a direct proportionality with stress relaxation. It is evident from the study that the concept of matrix stiffness induced mechanotransduction needs to be investigated in more details.

Keywords: stiffness, elasticity, Lamin A/C, immunocytochemistry, flow cytometry

List of Table and Figures

Table.1 List of parameters used for compression and stress relaxation study.

Fig. 1 Static mechanical forces sensed by cells. (A) Schematic of a cell on a stiff support, with attachment to the matrix via integrin-containing focal adhesions. These focal adhesions link to the actin–myosin cytoskeleton, which pulls on the matrix, senses resistance, and in turn responds by exerting increased tension (see arrows). (B) Schematic showing the measurement of elastic modulus *E*, which is the slope of the stress/strain curve.

Fig. 2 Pathways of force transmission from the extra cellular matrix to the nucleus. External forces can act on the cell through substrate strain or fluid shear stress. Integrins and other adhesion molecules physically couple the actin cytoskeleton to the extracellular matrix and can respond to extracellular ligands and intracellular signals. Cytoskeletal cross-linkers such as plectin can interconnect actin filaments, intermediate filaments, and microtubules. Plectin can also directly bind to nesprin-3 on the outer nuclear membrane, whereas the giant isoforms of nesprin-1 and -2 contain N-terminal actin binding domains. At the nuclear envelope, nesprins interact through their C-terminal KASH domain with SUN proteins, which cross the perinuclear space. At the inner nuclear membrane, SUN proteins can bind to lamins and other nuclear envelope proteins, which, in turn, can bind to DNA and chromatin, completing the physical link between the nucleus and the cytoskeleton. Cellular components are not drawn to scale.

Fig. 3 Mechanical characterization of PDMS substrates for sress relaxation paramrter. (a) Compression curve of different PDMS substrates. (b) Modulus of elasticity of PDMS substrates of varying crosslinker : PDMS base.

Fig. 4 Mechanical characterization of PDMS substrates for sress relaxation paramrter. (a) Stress relaxation curve of PDMS substrates (b) %SR of PDMS substrates of varying crosslinker : PDMS base.

Fig. 5 Representative image of anti-PCNA stained HaCaT cells on PDMS substrates. Scale bars 30μm.

Fig. 6 Cytogram showing G_1, S and G_2+M phases of the synchronized HaCaT cells after 7 days of serum starvation (DMEM without serum).

Fig. 7 Representative image of the cell cycle analysis of synchronized HaCaT cells cultured for 24h on PDMS substrates.

Fig. 8 Representative image for pERK ½ expression in HaCaT cells on PDMS substrates.

Fig. 9 Representative image for influence of PDMS substrate elasticity on the spreading of human keratinocytes (HaCaT cells).

Fig. 10 Representative image for focal adhesion formation on PDMS substrates visualized by pFAK397 staining (scale bars; 30 μm).

Fig. 11 Representative image for nuclear Lamin A/C expression in HaCaT cells cultures for 24h on PDMS substrates of varying stiffness (scale bars; 30 μm).

Chapter 1

INTRODUCTION

1. Introduction

Mechanotransduction describes the molecular mechanism by which the mechanical forces acting on the cells are converted to the biochemical signals and allows the cells to respond to the changes in mechanical forces. Cells adapt to the mechanical perturbations in the environment throughout the process of development. In this regard understanding mechanotransduction is important in various fields of biology, such as regenerative medicine, cancer and tissue bioengineering [1-2]. Matrix stiffness (matrix elasticity), one of the various mechanical forces acting on cells, is increasingly being appreciated as an important mediator of the cell behaviour. Matrix elasticity has been established as regulator of cell behaviours such as, cell spreading, proliferation, differentiation and migration for almost every mechanosensory cells [3-4]. Till now, very few substrates have been used to study the effect of substrate elasticity on cell behaviour. The majority of studies investigating substrate elasticity effect on cell behaviour have been carried out with Poly acrylamide (PAAM) substrate [5]. Now days, another cell substrate material has also emerged as model for matrix elasticity studies. The mechanical properties of Polydimethyl siloxane (PDMS) can also be easily modulated by varying the oligomer : crosslinker ratio. Moreover, recently two independent researches carried by Britta Trappmann et.al (2012) and Jianjun Li et.al (2014) have investigated the matrix elasticity effects on cell behaviour under the influence of material type (PAAM and PDMS) [6-7]. The studies were limited to the cell spreading behaviour but notably, both studies supported the common result that cell spreading behaviour was unaffected by the bulk stiffness of PDMS. Keeping the aforesaid perspective in mind, here, we have tried to look further into the effects of PDMS matrix stiffness on cytoskeletal reorganization through studying the cell spreading at interphase and during cell

division, proliferative behaviour of the cell, cell cycle regulation and nuclear mechanotransduction through nuclear lamin proteins.

In the present study, the effect of matrix stiffness and stress relaxation property (within physiological range) of PDMS has been investigated on transformed human keratinocytes (HaCaT cells). Effect of matrix stiffness and stress relaxation was studied for cytoskeletal reorganization, cell proliferation, cell cycle regulation and nuclear mechanotransduction of the HaCaT cells. The methods mainly used for this study were immunocytochemistry and flow cytometry techniques.

Chapter 2.

REVIEW OF LITERATURE

2. Review of literature

2.1 Role of matrix stiffness in cell proliferation and cytoskeletal reorganization

The concept of mechanotransduction evolved in the late 90s with the tensigrity model of mechanotransduction developed by Ingber [8-9]. Among the various mechanotransducers such as tension, compression, fluid shear stress and matrix stiffness; matrix stiffness has most importantly gained the interest of researchers. Matrix stiffness regulates the cell behaviour by controlling the cellular traction. Cellular traction forces are the cell generated tension through actomyosin contractility activity powered by ATP hydrolysis. This tension is then relayed to the ECM and the force exerted on the ECM is termed as cellular traction force [10]. The cellular traction force at the focal adhesions is in the range of tens of Newton. These forces are transmitted to the ECM through the focal adhesions and among them integrins are the primary mediators. Integrins are located at both the positions; at the ends of stress fibers and on the ECM. Thus, they physically connect the actin cytoskeleton to the ECM. Upon integrin activation a number of different cytoskeletal proteins like actin, talin and paxillin together with some signalling molecules such as focal adhesion kinases (FAK) are recruited and organized at the focal adhesion sites [11-12].

In vivo the mammalian cell commonly attaches to another similar cell or the ECM, and there they experience an elastic modulus in the order of 10 to 10,000 Pa [13] (Fig. 1). Accordingly, the cell morphology and functions depends on the substrate stiffness experienced by the cell. It has been shown that higher traction forces are generated by cells and they develop a broader and flatter morphology on stiff substrates in comparison to soft substrates [14]. Cell growth and proliferation rate has also been shown to be dependent on substrate stiffness [15-16]. Cells responds to both the mechanical and biochemical changes in ECM through the crosstalk between integrin surface receptors and the actin cytoskeleton. The integrin binds to different ECM molecules, this has been summarised in a review by Alam et al. (2007).

Hence, the specific integrin expression profile expressed by a cell dictates to which ECM substrate the cell can bind and the accompanying contents of integrin adheosomes dictates the downstream signalling events and hence the final cell behaviour and fate [17]. Integrin mostly recognises and binds to the Arg-Gly-Asp (RGD) motif of the proteins that are found in many other ECM proteins including integrins, laminin and vitronectin [18]. Integrin-dependent signalling events include the activation of ERK MAP kinases and FAK. However, not much information about these pathways activation and signal transduction is known in regard to physiologically relevant mechanical microenvironments.

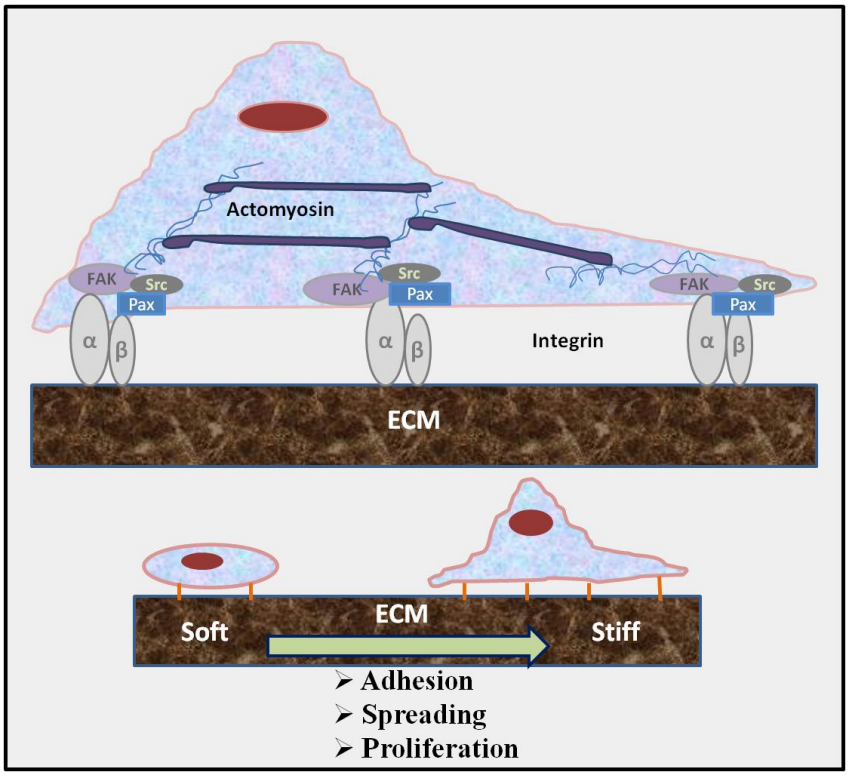


Fig. 1 Static mechanical forces sensed by cells and cell behaviour on soft and stiff matrices. Schematic of a cell on the matrix (ECM) attached via integrin-containing focal adhesions. The focal adhesions link to the actomyosin cytoskeleton, which pulls the matrix, senses the resistance, and responds by exerting traction forces on ECM. Below schematic showing the cell behaviour with variation in matrix stiffness.

2.2 Biophysical hallmarks of cell cycle progression

Cells connect to their external environment through the cytoskeleton in a complex manner. Cell cycle progression is guided by the external signals that cells receive from their environment. As such, the role of chemical signals has been well understood however, the importance of the physical signals has only recently been recognized to be prevalent and powerful. Now days, the importance of physical factors such as matrix stiffness has equally been realized to be playing an essential role in the cell cycle progression of most non transformed cell types. A number of adhesion-mediated signalling pathways and cell-cycle events regulating cell proliferation have been identified. It has been found that the regulation of cell cycle progression by matrix stiffness is widely conserved. A biophysical hallmark of the cell cycle progression is the ability of the cell to sense the stiffness of extracellular matrix, which suggests that matrix stiffness may be an important factor for the adhesion-dependent signalling events [19]. Collagen gels have been used to study the effect of matrix stiffness on integrin signalling and the cell cycle progression[20-21]. Reports are there that increased p21cip1 or p15INK4B are responsible for G1 phase arrest that happens when cells are seeded on soft collagen gels [22-23]. This also impairs the autophosphorylation of FAK, Rho activation and ERK activity [24]. The results shows that the such changes in the stiffness of matrix are similar to the effects seen upon complete blockade of the cell adhesion, but collagen gels are not applicable for such studies as they are less stiff than most physiological tissues (elastic moduli of 10–50 Pa versus 100–100,000 Pa) [13, 25]. Moreover, altering the stiffness of collagen gels also alters the ligand concentration as the stiffness can only be varied by varying collagen concentration. So, the observed effects cannot be primarily related to changes in the matrix elasticity alone. Control of substrate elasticity is achieved in best way by seeding cells on ECM-coated biocompatible hydrogels such as PAAM, because elasticity can be varied easily by varying the monomer : crosslinker ratio and this does not

effects the matrix concentration. However, PAAM gels can not be used in vivo because of toxicity issues related to acrylamide. It has been shown recently that the cell generated tension through the contracting cytoskeleton termed as traction forces can be used to sense stiffness of the ECM, which then affects the cytoskeletal organization and behaviour of the cell [26].

Mechanically cell cycle progression has a link with the cell cytoskeleton. The major component of cytoskeleton is the actin. Actin acts as the cellular scaffold and plays important mechanical roles such maintaining cell shape, intracellular vesicular transport, cell adhesion properties and the migration ability. Apart from these, actin cytoskeleton also plays important role in the chemical signal transduction. It was first hypothesized through inside-out signalling mechanism the cell cycle machinery controls the actin organization within the cell [27]. However, backward signalling where in actin organization within the cell controls cell cycle progression was proven to be important as well [28-29]. This can be easily shown through the experiments using drugs that interfere with the actin filament within the cell. Delayed progression of mitosis has been reported by using drugs such as cytochalasin D that interferes with actin depolymerisation [30].

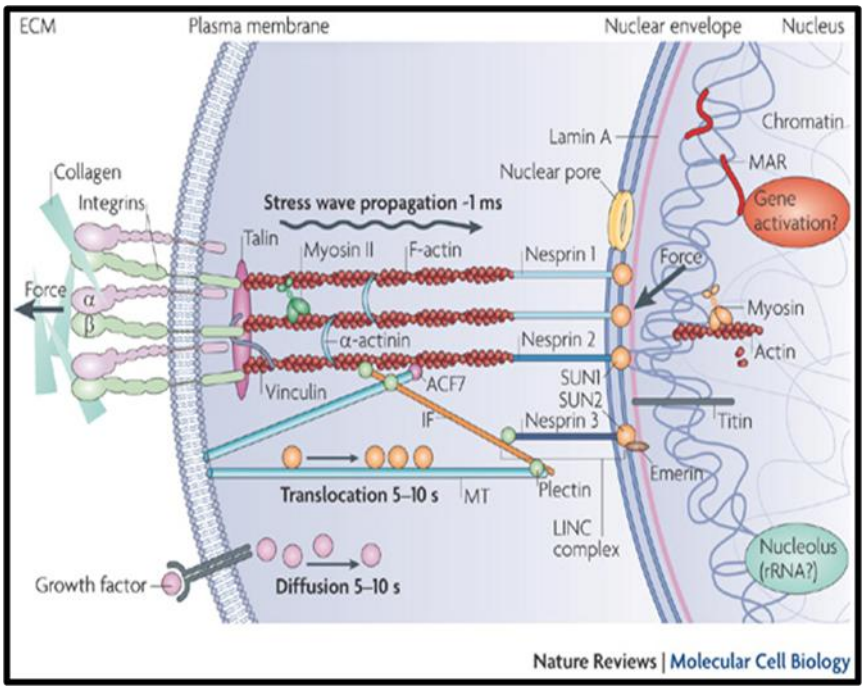
2.3 Nuclear Mechanotransduction

For most of the mechanotransduction events, cellular pathways for the downstream signalling in response to the force-sensed gene transcription have been well characterized. One of the mechanotransduction pathways is through opening of stretch sensitive ion channels. The most common ions for these ion channels is the calcium ion, its concentration inside the cell can change by ion influx and by ionic release from intracellular organelles. The changes in ionic concentrations show various downstream effects which include activation of signalling pathways that leads to the changes in gene transcription. However, recently it has been shown that the gene transcription is affected both by the cytoskeletally activated proteins along with

nuclear proteins that are mainly associated with nucleoskeletal structures such as Lamin A/C. There exist even other hypothesized mechanisms that correlate the nuclear shape to a mechanotransduction response of the cells. These mechanisms through which the changes in the nuclear cytoskeleton proteins or shape and size of nucleus in response to changes in mechanical signals directly affects the gene transcription leading to changes in cell behaviour may be termed as nuclear mechanotransduction.

The nucleus is known to be the largest and stiffest organelle with elasticity in the range of 0.1 to 10 kPa, which depends upon the cell type [31-32]. It is even having exposure to the extracellular as well as intracellular mechanical forces transmitted to nucleus via cytoskeleton from outside the cell and from force generated inside the cell. Moreover, the nucleus itself has been suggested as a the cellular mechanosensor, which causes conformational changes in chromatin structure and organization through changes in nuclear shape and directly affects the gene transcription [33]. Among nucleoskeletal proteins lamin proteins form the main nucleoskeletal component of the nuclear lamina and A-type lamins, which includes lamins A and C, during the developmental process are present at various levels in almost all differentiated cells. The A-type lamins are mainly responsible for the nuclear stiffness. Currently, only limited evidence exists that the gene transcription can be directly affected by the extracellular forces that are relayed to the nucleus and directly governs the DNA elements. The mechanism to carry the extracellular forces to the nuclear mechanotransduction is provided by the physical linkage among the cell cytoskeleton and the nuclear envelope surrounding the nucleus (Fig. 2). Physical associations have been found to associate extracellular integrins to the nuclear elements, and that extracellular forces can be relayed through cytoskeleton to the nucleus, resulting in the intranuclear deformations [34]. One such example is the interaction between cell cycle regulator protein retinoblastoma (Rb) and the nucleoplasmic lamin binding proteins and lamin A itself. It has been shown that the

DNA binding and the transcriptional activity of the activating protein (AP)-1 which affects the cellular proliferation is related to the expression levels of lamin A/C.



[Ref: Dahl K.N et al. Circulation research. 2008; 102: 1307-1318]

Fig.2 Pathways of force transmission from the extra cellular matrix to the nucleus. External forces acting on the cell through the ECM are mediated through the integrins and other adhesion molecules that physically link the actin cytoskeleton to the ECM and thus can respond to extracellular forces. Plectin protein interconnects the actin filaments, intermediate filaments, and microtubules. They also directly bind to nesprin-3 on the outer nuclear membrane, whereas nesprin-1 and -2 directly binds to the actin. At the nuclear envelope, nesprins interact with SUN proteins, which cross the perinuclear space. At the inner nuclear membrane, SUN proteins bind to lamins and other nuclear envelope proteins, which, in turn, bind to DNA and chromatin. This complets the physical link between the nucleus and the cytoskeleton.

Chapter 3.

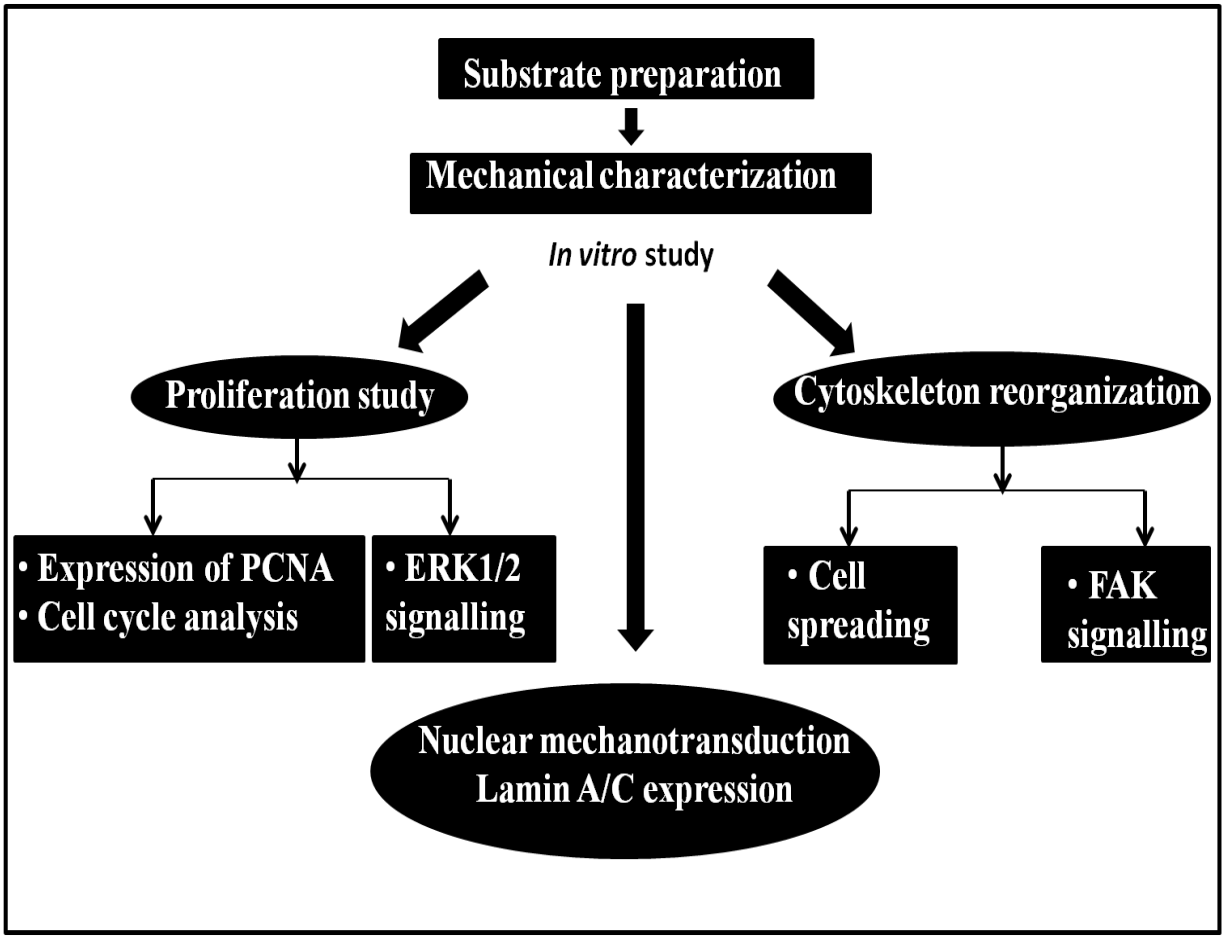
OBJECTIVES AND WORK PLAN

3. Objectives and work plan

3.1 Objectives of the work:

To investigate the influence of matrix stiffness and stress relaxation properties of the matrix on HaCaT cell proliferation, cytoskeleton reorganization and the nuclear mechano-transduction.

3.2 Plan of work:



3.3 Rationale of the study:

1. Choice of material for substrate preparation

To date very few materials have been used to study the effect of matrix elasticity on cell behaviour. The majority of them being PAAMs which can never be implanted in vivo because of the toxicity effects of acrylamide. To investigate whether substrate elasticity effects on the cell behaviour are independent of the material type we have chosen PDMS as the material for substrate preparation. Like PAAM, the mechanical properties of PDMS can also be easily varied by varying the crosslinker: base polymer ratio.

2. Range of matrix stiffness

To investigate whether the observed behaviour of cells on substrate of varying stiffness has any physiological relevance we have chosen the range of matrix stiffness (2.28 kPa to 0.69 k Pa) that lie within the physiological tissue stiffness range. Another reason is that in vivo generally cells do not experience large changes in matrix stiffness. So, we have chosen a small range of matrix elasticity.

3. Choice of cell type

Keratinocytes are mechanosensitive cells, in vivo the epidermis like various other tissues also experiences the variation in the stiffness of the several regions within the tissue. Moreover, so far not much study has been performed on effect of matrix stiffness on keratinocytes.

Chapter 4.

MATERIALS AND METHODS

4. MATERIALS REQUIRED

4.1 Materials: SYLGARD (R) 184 SILICONE ELASTOMER KIT was purchased from Dow corning India Private Ltd. Ethanol was brought from Merck, Mumbai, India. Isopropanol, Triton X-100, Dulbecco's Minimal Essential Medium (DMEM), Dulbecco's Phosphate Buffer Saline (DPBS), Trypsin-EDTA solution, Fetal Bovine serum, Antibiotic-Antimycotic solution and MTT assay kit were purchased from Hi-Media, Mumbai, India. The HaCaT cell line was procured from NCCS, Pune, India. TRITC Phalloidin, PCNA antibody, mouse primary pERK antibody, pFAK antibody and Lamin A/C antibody and secondary anti mouse Alexa flour 548 and 488 were procured from Sigma Aldrich, India.

METHODOLOGY

4.2 Preparation of PDMS substrates of varying elasticity

PDMS substrates of different stiffness were prepared by changing the polymer to crosslinker ratio. Three substrates were chosen for the work with the following polymer to crosslinker ratio, 65:1, 55:1 and 50:1. The polymer and crosslinker were mixed properly followed with degassing done through centrifugation at 5000 rpm for 15 min. Then the PDMS was poured into the wells and kept for baking at 65ºC for 4 hrs.

4.3 Mechanical characterization of PDMS substrates

Prepared substrates were characterized for compression and stress relaxation using TA.XT2i Texture Analyzer (Stable Micro Systems Ltd, Surrey, UK). Substrate stiffness was calculated in terms of the modulus of deformability using compression data (Table. 1). Modulus of deformability or elasticity is related to the slope of the force-time graph of the first

compression cycle within the linear range [35]. The percent stress relaxation is defined as the percent reduction in the force between F0 and F30 and is calculated by the formula:

$$\%SR = \frac{F_0 - F_{30}}{F_0} \times 100$$

Where, F_0 = peak force at a target distance of 5 mm after a trigger force of 5 g and

F_{30} = final force after holding the probe for 30 sec.

Type of study	Type of fixture	Pre test speed	Test speed	Post test speed	Mode of study
Stress relaxation	3mm probe	1mm/sec	1mm/sec	1mm/sec	Auto (Force) 5g,3mm
Compression	3mm probe	1mm/sec	1mm/sec	1mm/sec	Auto (Force) 3g,3mm

Table. 1 List of parameters used for compression and stress relaxation study.

In vitro studies

4.4 Cell culture

Human HaCaT cells were cultured in T-25 cell culture flasks (Eppendorf, Chennai, India) and maintained in DMEM media, having with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic solution and incubated at 37°C in an atmosphere containing 5% CO_2 [36].

4.5 Synchronization procedure

Synchronization of the HaCaT cells was done by culturing in the absence of serum. After the cells were grown to 100% confluent level in DMEM media containing 10% FBS for 4 days, then after one wash with DPBS the medium was replaced by serum-free high-glucose DMEM. The HaCaT cells were grown in serum-free medium for 7 days. The synchronized cells were trypsinized and were then seeded into T-25 culture flasks at a density of 4×10^3

cells/cm^2 in 10% FBS high-glucose DMEM. Cells were counted and their viability determined by trypan blue staining [36].

4.6 Cellular reorganization

Cytoskeletal rearrangement of cells on substrates of varying stiffness was visualized by staining the actin network and nucleus with TRITC-phalloidin and DAPI respectively. After 24 h incubation of cells on various substrates, cells were properly rinsed with ice cold phosphate buffer saline (PBS: pH 7.4) three times and fixed with paraformaldehyde (4% in PBS) for 15 min. Afterwards, cells were washed thrice with PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Fluorescent dye mixture was prepared by adding 6µl of DAPI and 8 µl of TRITC-Phalloidin in 2 ml of PBST (0.1 % Triton X-100 in PBS). 100µl if this dye mixture was added in each well and allowed to stand for 10 min. Unbound dye was removed by proper washing with PBS and samples were observed under confocal microscope (Olympus FluoView 1000). Images were further processed for analyzing the cellular reorganization using NIH ImageJ software in terms of cell spreading area and major to minor axis ratio.

4.7 Immunocytochemistry protocol

HaCaT cells were seeded into the wells containing PDMS (65:1, 55:1, 50:1) at a density of 5 × 10^4 cells/cm^2. The cells were cultured in these wells for different times. For immunocytochemical staining, the cells were fixed for 10 min at room temperature in 4% paraformaldehyde in PBS pH 7.4. They were then washed twice with ice cold PBS. For permeabilization, samples were incubated for 15 mins in PBS containing 0.1% Triton X-100 (PBST). Samples were again washed with PBS. To block unspecific binding of the antibodies cells were incubated for 1 hr with 1% BSA in PBST. 0.3M glycine was included in blocking buffer to bind with free aldehyde groups that would other bind to primary and secondary antibodies leading to high background. After blocking was over the cells were

incubated in primary antibodies (diluted in 1% BSA in PBST) for 1 hr at room temperature. The solution was decanted and the cells were washed with PBS for 3*5 mins. The cells were incubated in secondary antibodies (diluted in 1% BSA in PBST) for 1hr at room temperature and washed with PBS for 3*5 mins. For actin staining cells were incubated with TRITC-Phalloidin for 10 mins. Mouse monoclonal antibodies for FAK (pY397) at dilution of 1:500, ERK1/2 (pT202/pY204) at a dilution of 1:500 and Lamin A/C at a dilution of 1:500 were used as primary antibodies. Anti mouse Alexa 488 and Alexa 548 were used as secondary antibodies. For counter staining cells were incubated with 0.1 μg/ml DAPI for 2 mins. The cells were rinsed with PBS.

4.8 Cell cycle analysis through Flow cytometry

HaCaT cells were harvested by trypsinization and were washed twice with PBS. The cell density was adjusted to 2×10^6 cells/ml, afterwards 1 ml cell suspension was centrifuged at 2000 rpm for 10 min. The cell pellet was resuspended in 70% ice-cold ethanol and fixed for at least 24 h at –20 °C. After fixation the cells were centrifuged at 2000 rpm and suspended in 500 μl PI staining buffer (50 μg/ml PI, and 100 U/ml RNase) and stained for 15 min at room temperature. The samples were analysed using FACS Accuri C6 (BD Biosciences) and FACS Accuri C6 software.

Chapter 5.

RESULTS AND DISCUSSION

5. Results and Discussion

5.1 Mechanical characterization of PDMS substrates

Polydimethylsiloxane (PDMS) is widely used material for the study of the cell–substrate interactions because its mechanical properties can be easily varied within physiologically relevant ranges. Mechanical properties of PDMS can be easily modulated by changing the degree of crosslinking [37]. Mechanical properties of PDMS substrates were evaluated on the basis of 'modulus of elasticity' and 'stress relaxation' (SR). Here, with the increase in the ratio of PDMS elastomer base to crosslinker from 50:1 to 65:1, the elastic modulus of the substrate decreased from 2.28 ± 0.114 kPa to 0.69 ± 0.003 kPa (Fig. 3(b)). Modulus of elasticity was calculated from compression data of the PDMS substrates (Fig. 3(a)). Modulus of elasticity can be directly related to the stiffness; therefore we can use the term elasticity and stiffness interchangeably. Moreover, the %SR of the PDMS substrates increased from 25 ± 1% to 50±0.4 %. Notably, the variation in SR of the material was much higher in comparison to the variation in substrate elasticity. (Fig. 4(a) and 4(b)).

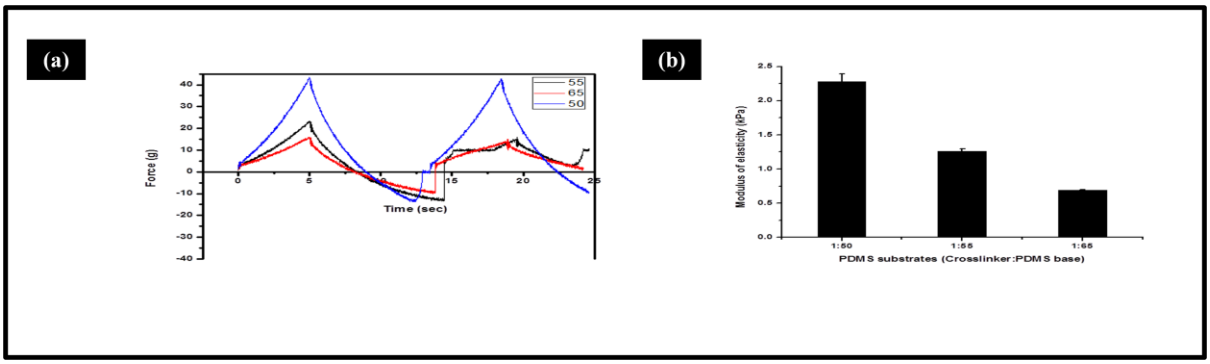


Fig. 3 Mechanical characterization of PDMS substrates for elasticity paramrter. (a) Compression curve of different PDMS substrates. (b) Modulus of elasticity of PDMS substrates of varying crosslinker : PDMS base.

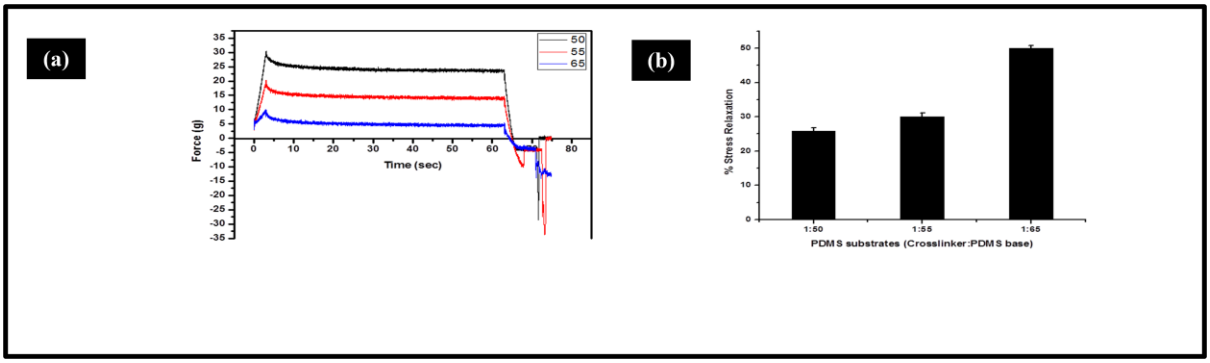


Fig. 4 Mechanical characterization of PDMS substrates for sress relaxation paramrter. (a) Stress relaxation curve of PDMS substrates (b) %SR of PDMS substrates of varying crosslinker : PDMS base.

5.2 Proliferation study of HaCaT cells cultured over PDMS substrates of varying stiffness

In general the cell proliferation is quantified by measuring the rate of DNA synthesis. DNA synthesis is known to take place in the S-phase of the cell cycle. Proliferating cell nuclear antigen (PCNA)/cyclin is an intranuclear polypeptide of 36 kDa molecular weight whose expression and synthesis is linked with the DNA synthesis and hence cell proliferation. Proliferating cell nuclear antigen (PNCA)/cyclin is found in both normal and transformed proliferating cells [38]. The proliferation of HaCaT cells cultured over various PDMS substrates (1:50, 1:55, 1:65) was determined by immunofluorescence microscopy of cultured cells with monoclonal anti-PCNA. In all the cases, significant number of HaCaT cells showed nuclear localization of the anti-PCNA irrespective of the variation in matrix stiffness (Fig. 5). Moreover, there was not much variation in the number of PCNA positive cells on various PDMS substrates. This indicates that the variation in matrix stiffness of PDMS in the range of 2.28 kPa to 0.69 kPa does not significantly effects the proliferation of HaCaT cells.

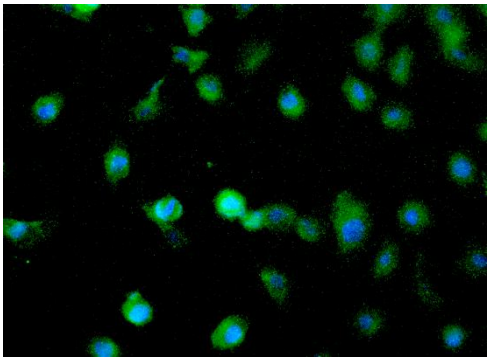


Fig. 5 Representative image of anti-PCNA stained HaCaT cells on PDMS substrates.

5.3 Cell cycle analysis of synchronized HaCaT cells cultured over PDMS substrates of varying stiffness

Flow cytometry is routinely used for analysis of cell cycle. Analysis of cell cycle is helpful in studying the cell proliferation by determining the fraction of cells in G_1, S and G_2+M phases of the cell cycle [39]. DNA synthesis takes place in S phase of the cell cycle and is associated with the rate of cell proliferation. After serum starvation for 7 days 70% of the cell population stays in G_1 phase of the cell cycle with merely 18% cells in the S and G2+M phase of the cell cycle (Fig. 6). Here, HaCaT cells synchronized through serum starvation are also cultured on PDMS substrates of different stiffness (2.28 kPa to 0.69 kPa) for 24h. The cytograms show that the HaCaT cells in all the cases have approximately 30% of the cell fraction in S phase of the cell cycle (Fig. 7). This indicates that the number of cells proliferating in all the cases is nearly same without any effect of the matrix stiffness of the PDMS substrates on HaCaT cells. This result confirms the proliferation study through anti-PCNA. Moreover, after 24h of synchronization not more than 10-14% of the cells are in G_2+M phase of the cell cycle while the number of cells in S-phase has doubled in comparison to the synchronized cells.

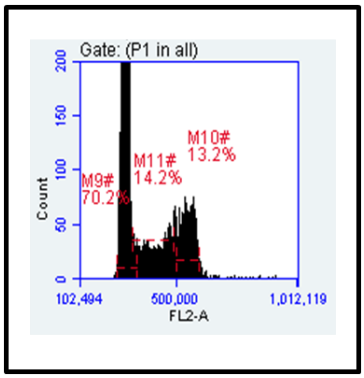


Fig. 6 Cell cycle analysis of synchronized HaCaT cells. Cytogram showing G_1, S and G_2+M phases of the synchronized HaCaT cells after 7 days of serum starvation (DMEM without serum).

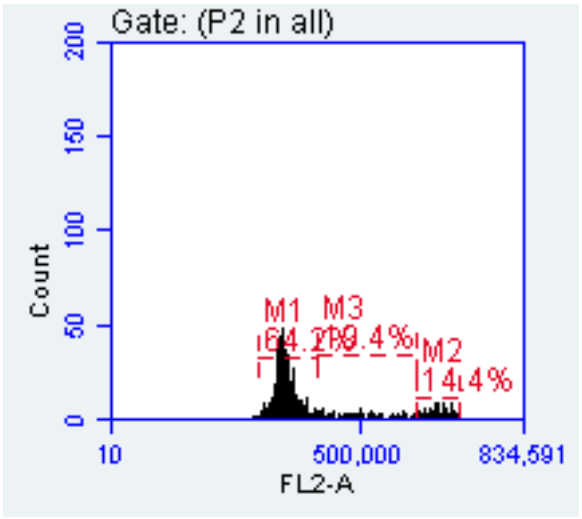


Fig. 7 Representative image for cell cycle analysis of synchronized HaCaT cells cultured for 24h on PDMS substrates

5.4 Expression of pERK1/2 in HaCaT cell cultured over PDMS substrates of varying stiffness

The MAP kinase-signaling pathways are significant for the proliferative response of various cells towards mechanical forces. Expression of ERK1/2 is indispensable for cell proliferation and survival of various cell types [40]. To confirm the results of proliferation study of HaCaT cells on PDMS substrates of varying stiffness, activation of ERK1/2 was checked in the cells. Expression of pERK1/2 was confirmed through immunofluorescence. On all the PDMS

substrates, HaCaT cells showed similar expression profile for pERK1/2 (Fig. 8). This showed same level of activation of ERK1/2 in the HaCaT cells cultured over PDMS substrates of varying stiffness in the range of 2.28 kPa to 0.69 kPa. This was further confirmed by the intensity profile of pERK1/2 expression obtained through flow cytometry. HaCaT cells on all the PDMS substrates were found to give fluorescence intensity in the range of 10^4 to 10^5 on a log scale, with no significant variation in mean intensity value for cells cultured on PDMS substrates of different matrix stiffness (Fig. 9). Moreover there is no significant variation in expression of pERK1/2 in cells cultured over PDMS substartes and glass (taken as control). This further confirms that the proliferation of HaCaT cells is not affected by the matrix stiffness of PDMS substrates in the range of 2.28 kPa to 0.69 kPa.

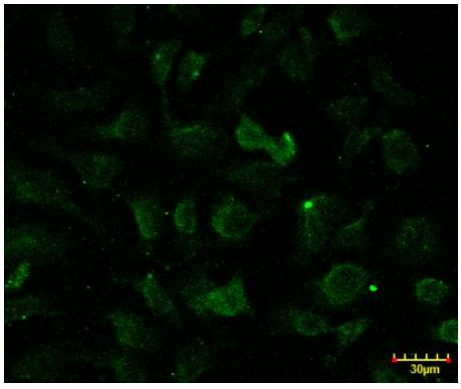


Fig. 8 Representative image for pERK ½ expression in HaCaT cells on PDMS substrates.

5.5 Cytoskeletal reorganization of HaCaT cells on PDMS matrices of varying stiffness

This study aimed to analyze the changes in cell area and shape as a result of cell binding to extracellular matrix of varying stiffness. Here, we have focused on the initial process of cell spreading that follows cell attachment to matrix during interphase and the change of shape and cellular area in dividing cells considering the metaphase as the divisional phase. Thus,

the cell ‘shape’ changes are defined here in terms of changes in the cell spreading areas, as determined by image analysis using NIH Image J software. The main component of the cytoskeleton is the actin microfilament. Microtubules are also important component of cytoskeleton that drives cell division and decides the cell shape and area during division is mostly dependent upon the microtubules. Cell spreading was found to be unaffected by the PDMS stiffness in the range used. Cell area was calculated using Image J software. The difference between the spreading area of HaCaT cells cultured on PDMS substrates of different stiffness is not significant ($p<0.05$) both during initial process of cell spreading (interphase) and during division (metaphase) (Fig. 10). These results are in agreement with the Britta Trappmann et.al (2012) which also showed that cell spreading is unaffected by PDMS stiffness.

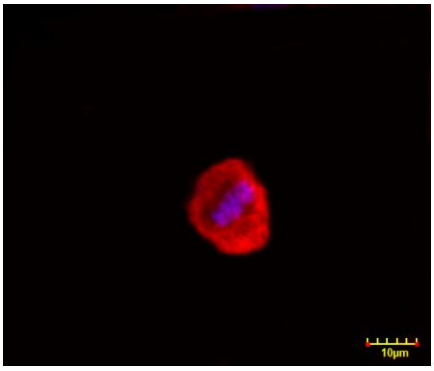


Fig. 9 Representative image for influence of PDMS substrate elasticity on the spreading of human keratinocytes (HaCaT cells)

5.6 Cell adhesion mediated signalling : expression of pFAK^{397}

Integrins are the primary receptors for ECM ligands and are located in focal adhesion points. They provide a link between ECM and the actin cytoskeleton and thus control several signalling pathways. The implication of integrin β1 in mechanical signalling pathways has been demonstrated in various cell types including keratinocytes [40]. Activation of focal adhesion kinase (FAK) through the clustering of integrins is required for MAP kinase activation upon generation of force. Focal adhesion formation on PDMS substrates was

visualised by anti-pFAK immuno staining. Level of focal adhesion formation was observed to be nearly same on all PDMS substrates indicating no effect of PDMS stiffness on mechanical signal transduction through integrins (Fig. 11). This also supports the insignificant variation of pERK ½ expressions which lies downstream to the FAK and relies on the activation of FAK for its activation through phosphorylation. Thus, the expression profile of FAK-ERK mediated signalling pathway validates that neither the matrix stiffness of PDMS within this range nor the SR has any effect on the cytoskeleton reorganization and proliferation of HaCaT cells.

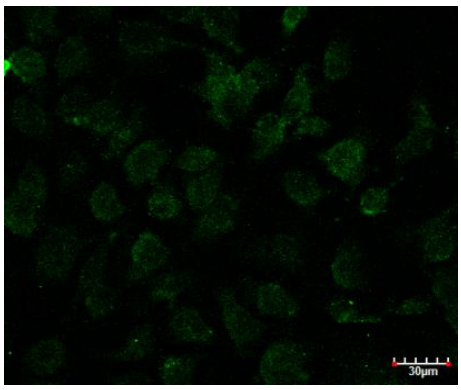


Fig. 10 Representative image for focal adhesion formation on PDMS substrates visualized by pFAK397 staining (scale bars; 30 µm).

5.7 Nuclear mechanotransduction

For most of the mechanotransduction events, cellular pathways for the downstream signalling in response to the force-sensed gene transcription have been well characterized.

However, recently it has been shown that the gene transcription is affected both by the cytoskeletally activated proteins along with nuclear proteins that are mainly associated with nucleoskeletal structures such as Lamin A/C. These mechanisms through which the changes in the nuclear cytoskeleton proteins or shape and size of nucleus in response to changes in mechanical signals directly affects the gene transcription leading to changes in cell behaviour may be termed as nuclear mechanotransduction. [41]. So in order to investigate

the effect of PDMS matrix stiffness on nuclear mechanotransduction expression of anti-LaminA/C was studied. Interestingly, it was found that unlike cell spreading and proliferation the Lamin A/C levels were affected by the PDMS elasticity. The expression of LaninA/C decreased with increasing matrix stiffness (Fig. 12). However, this observation is in contrast with Joe Swift et.al (2013) which says that Lamin A levels scales with tissue stiffness [41]. This might be explained based on changes in stress relaxation of PDMS substrates that followed an opposite trend to that of PDMS elasticity. Moreover, SR parameter had no effect on cell adhesion and proliferation so this has to be checked that the signalling to nucleus is carried through intermediate filaments other than integrin-actin pathway which is not sensitive to PDMS elasticity. One proposed mechanism of force transmission to nucleus may be through keratin intermediate filaments owing to the unique cytoskeleton of keratinocytes [40].

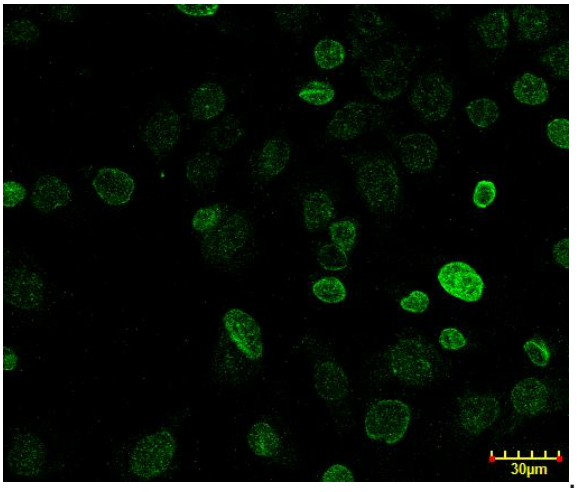


Fig. 11 Representative image for nuclear Lamin A/C expression in HaCaT cells cultures for 24h on PDMS substrates of varying stiffness (scale bars; 30 µm).

Chapter 6.

CONCLUSION AND FUTURE WORK

6.1 Conclusion

In conclusion, the study showed that matrix stiffness (2.28 k Pa to 0.69 k Pa) of PDMS and its associated stress relaxation do not mechanotransduce HaCaT cells. However, these set of parametes induce the nuclear mechanotransduction. A probable cause could be the rate of stress relaxation which influences cellular perception of basal dynamics. However the last point needs to be studied in details. Further studies are required to explain the reason for the relation between lamin A/C expression and matrix elasticity of PDMS. An integrative and systematic approach is required to test the material properties that influences the effect of matrix stiffness on cell behaviour.

6.2 Future Work:

[i] To investigate the pathways in HaCaT cells that leads to the signal transduction to lamin A/C due to changes in matrix elasticity.

[ii] To systematically study the various properties of the PDMS substrates that influence the effect of matrix elasticity on cell behaviour.

Chapter 7.

BIBLIOGRAPHY

References:

1. **DuFort, C.C., M.J. Paszek, and V.M. Weaver, *Balancing forces: architectural control of mechanotransduction.* Nature reviews Molecular cell biology, 2011. 12(5): p. 308-319.**
2. **Yang, X.-f., Y. Xin, and L.-l. Mao, *Clinicopathological significance of PTEN and caspase-3 expressions in breast cancer.* Chinese Medical Sciences Journal, 2008. 23(2): p. 95-102.**
3. **Hadjipanayi, E., V. Mudera, and R. Brown, *Close dependence of fibroblast proliferation on collagen scaffold matrix stiffness.* Journal of tissue engineering and regenerative medicine, 2009. 3(2): p. 77-84.**
4. **Discher, D.E., P. Janmey, and Y.-l. Wang, *Tissue cells feel and respond to the stiffness of their substrate.* Science, 2005. 310(5751): p. 1139-1143.**
5. **Wong, J.Y., J.B. Leach, and X.Q. Brown, *Balance of chemistry, topography, and mechanics at the cell–biomaterial interface: issues and challenges for assessing the role of substrate mechanics on cell response.* Surface Science, 2004. 570(1): p. 119-133.**
6. **Li, J., D. Han, and Y.-P. Zhao, *Kinetic behaviour of the cells touching substrate: the interfacial stiffness guides cell spreading.* Scientific reports, 2014. 4.**
7. **Trappmann, B., et al., *Extracellular-matrix tethering regulates stem-cell fate.* Nature materials, 2012. 11(7): p. 642-649.**
8. **Ingber, D.E., *Tensegrity I. Cell structure and hierarchical systems biology.* Journal of Cell Science, 2003. 116(7): p. 1157-1173.**
9. **Ingber, D.E., *Tensegrity II. How structural networks influence cellular information processing networks.* Journal of Cell Science, 2003. 116(8): p. 1397-1408.**
10. **Kolega, J., L.W. Janson, and D.L. Taylor, *The role of solation-contraction coupling in regulating stress fiber dynamics in nonmuscle cells.* The Journal of cell biology, 1991. 114(5): p. 993-1003.**
11. **Zamir, E. and B. Geiger, *Molecular complexity and dynamics of cell-matrix adhesions.* Journal of Cell Science, 2001. 114(20): p. 3583-3590.**
12. **Riveline, D., et al., *Focal contacts as mechanosensors externally applied local mechanical force induces growth of focal contacts by an mdia1-dependent and rock-independent mechanism.* The Journal of cell biology, 2001. 153(6): p. 1175-1186.**
13. **Bao, G. and S. Suresh, *Cell and molecular mechanics of biological materials.* Nature materials, 2003. 2(11): p. 715-725.**
14. **Yeung, T., et al., *Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion.* Cell motility and the cytoskeleton, 2005. 60(1): p. 24-34.**

15. **Engler, A.J., et al., *Myotubes differentiate optimally on substrates with tissue-like stiffness pathological implications for soft or stiff microenvironments.* The Journal of cell biology, 2004. 166(6): p. 877-887.**

16. **Hsiong, S.X., et al., *Differentiation stage alters matrix control of stem cells.* Journal of Biomedical Materials Research Part A, 2008. 85(1): p. 145-156.**

17. **Hemler, M.E. and R.R. Lobb, *The leukocyte [beta] 1 integrins.* Current opinion in hematology, 1995. 2(1): p. 61-67.**

18. **Ruoslahti, E., *Fibronectin and its integrin receptors in cancer.* Advances in cancer research, 1999. 76: p. 1-20.**

19. **Klein, E.A., et al., *Cell-cycle control by physiological matrix elasticity and in vivo tissue stiffening.* Current Biology, 2009. 19(18): p. 1511-1518.**

20. **Fringer, J. and F. Grinnell, *Fibroblast Quiescence in Floating or Released Collagen Matrices CONTRIBUTION OF THE ERK SIGNALING PATHWAY AND ACTIN CYTOSKELETAL ORGANIZATION.* Journal of Biological Chemistry, 2001. 276(33): p. 31047-31052.**

21. **Rosenfeldt, H. and F. Grinnell, *Fibroblast quiescence and the disruption of ERK signaling in mechanically unloaded collagen matrices.* Journal of Biological Chemistry, 2000. 275(5): p. 3088-3092.**

22. **Koyama, H., et al., *Fibrillar collagen inhibits arterial smooth muscle proliferation through regulation of Cdk2 inhibitors.* Cell, 1996. 87(6): p. 1069-1078.**

23. **Wall, S.J., Z.-D. Zhong, and Y.A. DeClerck, *The cyclin-dependent kinase inhibitors p15INK4B and p21CIP1 are critical regulators of fibrillar collagen-induced tumor cell cycle arrest.* Journal of Biological Chemistry, 2007. 282(33): p. 24471-24476.**

24. **PaszeK, M.J., et al., *Tensional homeostasis and the malignant phenotype.* Cancer cell, 2005. 8(3): p. 241-254.**

25. **Willits, R.K. and S.L. Skornia, *Effect of collagen gel stiffness on neurite extension.* Journal of Biomaterials Science, Polymer Edition, 2004. 15(12): p. 1521-1531.**

26. **Fletcher, D.A. and R.D. Mullins, *Cell mechanics and the cytoskeleton.* Nature, 2010. 463(7280): p. 485-492.**

27. **Roca, J. and J.C. Wang, *The capture of a DNA double helix by an ATP-dependent protein clamp: a key step in DNA transport by type II DNA topoisomerases.* Cell, 1992. 71(5): p. 833-840.**

28. **Assoian, R.K. and X. Zhu, *Cell anchorage and the cytoskeleton as partners in growth factor dependent cell cycle progression.* Current opinion in cell biology, 1997. 9(1): p. 93-98.**

29. **Théry, M. and M. Bornens, *Cell shape and cell division.* Current opinion in cell biology, 2006. 18(6): p. 648-657.**

30. **Engelman, J.A., et al., *MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling.* Science, 2007. 316(5827): p. 1039-1043.**

31. **Kha, H., et al., *Stiffness properties for Nucleus standard straight and contour electrode arrays.* Medical engineering & physics, 2004. 26(8): p. 677-685.**

32. **Caille, N., et al., *Contribution of the nucleus to the mechanical properties of endothelial cells.* Journal of biomechanics, 2002. 35(2): p. 177-187.**

33. **Dahl, K.N., A.J. Ribeiro, and J. Lammerding, *Nuclear shape, mechanics, and mechanotransduction.* Circulation research, 2008. 102(11): p. 1307-1318.**

34. **Wang, N., J.D. Tytell, and D.E. Ingber, *Mechanotransduction at a distance: mechanically coupling the extracellular matrix with the nucleus.* Nature reviews Molecular cell biology, 2009. 10(1): p. 75-82.**

35. **Lewandowicz, G., et al., *Influence of potato starch oxidation on texture, and rheological behaviour of some sweet desserts.* Polish journal of food and nutrition sciences, 2003. 12(2): p. 31-36.**

36. **Pivarcsi, A., et al., *Serum factors regulate the expression of the proliferation-related genes α5 integrin and keratin 1, but not keratin 10, in HaCaT keratinocytes.* Archives of dermatological research, 2001. 293(4): p. 206-213.**

37. **Brown, X.Q., K. Ookawa, and J.Y. Wong, *Evaluation of polydimethylsiloxane scaffolds with physiologically-relevant elastic moduli: interplay of substrate mechanics and surface chemistry effects on vascular smooth muscle cell response.* Biomaterials, 2005. 26(16): p. 3123-3129.**

38. **Kurki, P., K. Ogata, and E. Tan, *Monoclonal antibodies to proliferating cell nuclear antigen (PCNA)/cyclin as probes for proliferating cells by immunofluorescence microscopy and flow cytometry.* Journal of immunological methods, 1988. 109(1): p. 49-59.**

39. **Gray, J., et al., *Cell cycle analysis using flow cytometry.* International Journal of Radiation Biology, 1986. 49(2): p. 237-255.**

40. **Reichelt, J., *Mechanotransduction of keratinocytes in culture and in the epidermis.* European journal of cell biology, 2007. 86(11): p. 807-816.**

41. **Swift, J., et al., *Nuclear lamin-A scales with tissue stiffness and enhances matrix-directed differentiation.* Science, 2013. 341(6149): p. 1240104.**